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Comparison of Adeno-Associated Virus Pseudotype 1, 2, and 8 Vectors Administered by Intramuscular Injection in the Treatment of Murine Phenylketonuria

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Abstract

Phenylketonuria (PKU) is caused by hepatic phenylalanine hydroxylase (PAH) deficiency and is associated with systemic accumulation of phenylalanine (Phe). Previously we demonstrated correction of murine PKU after intravenous injection of a recombinant type 2 adeno-associated viral vector pseudotyped with type 8 capsid (rAAV2/8), which successfully directed hepatic transduction and *Pah* gene expression. Here, we report that liver PAH activity and phenylalanine clearance were also restored in PAH-deficient mice after simple intramuscular injection of either AAV2 pseudotype 1 (rAAV2/1) or rAAV2/8 vectors. Serotype 2 AAV vector (rAAV2/2) was also investigated, but long-term phenylalanine clearance has been observed only for pseudotypes 1 and 8. Therapeutic correction was shown in both male and female mice, albeit more effectively in males, in which correction lasted for the entire period of the experiment (>1 year). Although phenylalanine levels began to rise in female mice at about 8–10 months after rAAV2/8 injection they remained only mildly hyperphenylalaninemic thereafter and subsequent supplementation with synthetic tetrahydrobiopterin resulted in a transient decrease in blood phenylalanine. Alternatively, subsequent administration of a second vector with a different AAV pseudotype to avoid immunity against the previously administrated vector was also successful for long-term treatment of female PKU mice. Overall, this relatively less invasive gene transfer approach completes our previous studies and allows comparison of complementary strategies in the development of efficient PKU gene therapy protocols.

Introduction

Phenylketonuria (PKU) is an autosomal recessive genetic disorder with an average incidence of roughly 1 case in 10,000 Caucasian live births (OMIM 261600). It is caused primarily by deficiency of the hepatic enzyme phenylalanine hydroxylase (PAH; EC 1.14.16.1), responsible for converting phenylalanine to tyrosine, using molecular oxygen and tetrahydrobiopterin (BH_4) as a necessary cofactor for its catalytic activity (Scriver and Kaufman, 2001). This conversion is the rate-limiting step in phenylalanine (Phe) catabolism in the liver. PAH deficiency due to mutations in the gene that encodes PAH leads to hyperphenylalaninemia (HPA), a dramatic increase in blood Phe concentration from <0.12 to >1.2 m*M* (the equivalent of <2 to >20 mg/dl). The inability to degrade Phe present in dietary protein leads to the excretion of urinary phenylalanine, phenylpyruvate, and phenylacetate. Mild forms of PKU accumulate Phe in the blood at levels of 0.6 to 1.2 m*M*. High levels of accumulated Phe in patients with PKU are toxic to the human body if left untreated, and are associated with an abnormal phenotype presenting with growth failure, microcephaly, seizures, and mental retardation (Donlon *et al.*, 2008). HPA can also be caused by the absence of the BH_4 cofactor due to deficiency of cofactor biosynthesis and regeneration, which leads to a diverse group of neurometabolic diseases (Blau *et al.*, 2001; Thöny and Blau, 2006). The biochemistry and genetics of PKU are well characterized, with more than 500 disease-causing mutations identified in the human *PAH* gene spread over the entire 13 exon-containing gene (see also the Phenylalanine Hydroxylase Locus Knowledgebase [PAHdb], www.pahdb.mcgill.ca) (Scriver *et al.*, 2003). Moreover, PKU has been detected in most Western countries for many decades by newborn screening programs (Guthrie, 1996).

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HPA during pregnancy can produce PKU-like symptoms in genetically unaffected offspring, termed maternal PKU syndrome, which is associated with severe birth defects such as microcephaly, congenital heart disease, and low birth weight (Levy and Ghavami, 1996; Guttler *et al.*, 2003). The current established treatment for PKU consists of life-long dietary Phe restriction, which is challenging in practice as it is demanding in schedule, unpleasant in taste, carries risk for nutritional deficiencies, and is a substantial psychosocial burden, associated with reduction in quality of life (Harding, 2000; National Institutes of Health Consensus Development Panel, 2001). Furthermore, it was reported that decline in intellectual function (Koch *et al.*, 1984), behavioral performance (Koch *et al.*, 2002), and severe emotional dysfunction, including attention deficit disorder (Antshel and Waisbren, 2003), depression, and anxiety (Waisbren and Levy, 1991), are often observed in noncompliant patients with PKU.

Because of these shortcomings, the focus of PKU research has shifted since the beginning of the twenty-first century to the improvement of current therapy and development of alternative options (Sarkissian *et al.*, 2009). With the progress in gene delivery, particularly with the advent of adeno-associated virus (AAV)-based vectors (Wu *et al.*, 2006; Buning *et al.*, 2008; Schultz and Chamberlain, 2008; Zincarelli *et al.*, 2008), gene therapy has emerged as an attractive alternative to meet the particularly challenging issue of long-term treatment in PKU. Successful correction of murine HPA using recombinant type 2 AAV vectors pseudotyped with serotype 2 or 5 capsid (rAAV2/2 or rAAV2/5) has been achieved in several laboratories (Laipis *et al.*, 2004; Mochizuki *et al.*, 2004; Oh *et al.*, 2004). However, in these AAV experiments, extremely high doses of viral vectors (10^{13} to 10^{14} vector genomes per mouse) were necessary for efficient reduction in blood Phe. Moreover, the therapy was less effective in female mice. In one published study, efficacy was observed to last 40 weeks in male mice but only 10 weeks in females (Mochizuki *et al.*, 2004). Gender-dependent differences in liver transduction with AAV vectors have been reported in many other studies (Davidoff *et al.*, 2003; Grimm *et al.*, 2003; Berraondo *et al.*, 2006; Ogura *et al.*, 2006; Voutetakis *et al.*, 2007; Ho *et al.*, 2008) and could complicate human clinical trials, with important implications for the prevention of maternal PKU. Furthermore, other factors, such as stability of the PAH protein (Harding *et al.*, 2006a), subphysiological hepatic concentrations of tetrahydrobiopterin (BH_4, an essential cofactor for PAH) (Chen *et al.*, 2007), or differences in intestinal absorption and transport of Phe (Scriver and Waters, 1999), may also contribute to the lower Phe tolerance observed in treated PKU female mice. In this regard, experiments with nonviral vectors designed for stable liver-directed genomic integration of a PAH expression cassette (Chen and Woo, 2007) or with AAV vectors pseudotyped with serotype 8 capsid (rAAV2/8) (Ding *et al.*, 2006b; Harding *et al.*, 2006b) also indicated that a higher therapeutic threshold is required for long-term correction of PKU in females. Recombinant AAV2/8 has been reported to be 10 to 100 times more effective in liver than rAAV2/2 and rAAV2/5, respectively (Sarkar *et al.*, 2004). In addition to the role of the AAV capsid in gene transfer, the number of hepatocytes that are successfully transduced, even when high doses of AAV vector are used, is highly influenced by the routes of administration for AAV delivery (Berraondo *et al.*, 2006; Inagaki *et al.*, 2006). Indeed, our previous study showed a more robust and longer lasting correction of hyperphenylalaninemia in both male and female PKU mice when rAAV2/8 vectors were delivered via the portal vein rather than by the tail vein (Ding *et al.*, 2006a). In that experiment, expression of the PAH gene was driven by the constitutive or ubiquitous cytomegalovirus (CMV) enhancer–chicken β-actin (CBA) promoter. Although therapeutic corrections up to 53 weeks (for more than 1 year) have been observed for some animals, we also noticed a loss of effectiveness starting at about week 40 in female mice. Together, these observations emphasize the need to further extend the therapeutic persistence achieved with rAAV2/8 vectors and to find alternative AAV serotype vectors if repeated gene vector delivery is required.

In this study, we performed a dose–response and time course analysis of the efficiency of recombinant AAV2/1, AAV2/2, and AAV2/8 vectors, expressing the murine *Pah* gene from the CBA promoter, and delivered by direct intramuscular injection rather than intravenously, to mediate liver transduction and correct hyperphenylalaninemia in Pah^{enu2} mice, a model of human PKU. The superiority of rAAV2/1 and rAAV2/8 vectors over rAAV2/2 vector in achieving long-term therapeutic correction was reaffirmed. More importantly, a therapeutic effect was observed in both male and female treated mice, albeit more effective in males. For treated female mice in which blood Phe levels had risen again several weeks after gene transfer, supplementation with BH_4 cofactor led to transient restoration of blood Phe to near normal levels. Alternatively, intramuscular injection of rAAV2/8 vector extended therapeutic correction in females previously exposed to rAAV2/1 vector.

Materials and Methods

Plasmid vectors and recombinant AAV production

The cloning strategy for the construction of plasmid pAAV2-PKU5 has been previously described (Ding *et al.*, 2006a). Briefly, the transgene expression cassette flanked by two inverted terminal repeats in pAAV2-PKU5 was designed with the CMV enhancer–chicken β-actin (CBA) promoter, the mouse 1.4-kb *Pah* cDNA, the woodchuck posttranscriptional regulatory element (WPRE), and the simian virus 40 (SV40) polyadenylation signal sequence. Recombinant AAV2 vectors pseudotyped with serotype 1 or 8 capsid proteins were produced in an adenovirus-free system in HEK 293 cells, using a three-plasmid transfection protocol with the pAAV2-PKU5 plasmid, the adenovirus helper plasmid pBS-E2A-VA-E4 (provided by H. Büeler [Paterna *et al.*, 2004]), and a pAAV packaging plasmid expressing the *rep* and *cap* genes. For pseudotyping with serotype 1 and 8 capsid proteins, plasmids p5E18RXCI and p5E18-VD2/8 were used, respectively (provided by J.M. Wilson [Gao *et al.*, 2002]). For the production of recombinant AAV2 serotype 2 vectors, a two-plasmid transfection protocol was used with plasmids pAAV2-PKU5 and pDG, the latter carrying the AAV2 *rep* and *cap* genes, as well as adenovirus helper genes (provided by J.A. Kleinschmidt [Grimm *et al.*, 1998]). All vectors were purified by two rounds of cesium chloride gradient centrifugation to provide a uniform purification method. After the second centrifugation, the peak fractions, as determined by semiquantitative polymerase chain reaction (PCR), were

dialyzed for three rounds against sterile phosphate-buffered saline (PBS), and concentrated with Amicon Ultra filters (Millipore, Billerica, MA) (Grieger *et al.*, 2006). The physical particle titers were determined by TaqMan analysis of the WPRE sequence with the following primers and probe (Potter *et al.*, 2002; Grieger *et al.*, 2006): forward primer, 5′-CCGTTGTCAGGCAACGTG-3′; reverse primer, 5′-AGCTGACAGGTGGTGGCAAT-3′; probe, 5′-FAM-TGCTGACGCAACCCCCACTGGT-TAMRA-3′. A standard curve was generated by dilution of vector plasmids in salmon sperm DNA (40 ng/ml) to increase the stability of plasmid dilutions.

Animal experiments

Animal experiments were carried out in accordance with the State Veterinary Office of Zürich (Zürich, Switzerland) and Swiss law on animal protection, the Swiss Federal Act on Animal Protection (1978), and the Swiss Animal Protection Ordinance (1981). All animal studies were approved by the Cantonal Veterinary Office (Zürich, Switzerland) and the Cantonal Committee for Animal Experiments (Zürich, Switzerland). PAH-deficient C57BL/6-*Pah*^{enu2} ("PKU") mice were homozygous for the same *Pah* mutation as described for the original BTBR-*Pah*^{enu2} strain (McDonald and Charlton, 1997; Ding *et al.*, 2006a). All mice were maintained on standard mouse chow, and 8- to 10-week-old male and female PKU mice were selected for viral injection. Fifty microliters of recombinant AAV vector suspension was injected intramuscularly through the skin into both gastrocnemius muscles in less than 1 min (about 0.025 ml/min); mice were not included in this study if blood was present in the syringe on withdrawal, or if the injected mice showed signs of bleeding. Monitoring of blood Phe collected from tail veins was carried out as described earlier (Ding *et al.*, 2008). For cofactor administration, BH_4 from Schircks Laboratories (Jona, Switzerland) was dissolved in 1% ascorbic acid, pH 7, to a concentration of 10–100 m*M* and administered to mice (between 16 and 32 mg of BH_4 per kilogram body weight) by intraperitoneal injection, using a 27-gauge needle on a 1-ml disposable syringe. Note that when the first cohort of animals was injected with a dose of 32 mg of BH_4 per kilogram body weight, we noticed moderate signs of weakness, passivity, and sluggishness in these animals; however, mice receiving 16 mg of BH_4 per kilogram body weight remained asymptomatic after BH_4 treatment. The amount of BH_4 given here is comparable to human doses for oral loading tests, which are between 20 to 40 mg of BH_4 per kilogram body weight. Tissue BH_4 content was determined 24 hr after intraperitoneal injection as described (Ding *et al.*, 2006a, 2008).

PAH enzyme assay and immunoblotting analysis

PAH enzyme activity in liver and muscle homogenates was measured according to a published method (Thöny *et al.*, 2004) with minor modifications of the homogenization procedure. Briefly, frozen harvested tissues were ground to powder in liquid nitrogen, suspended in 1 vol (muscle) or 5 vol (liver) of cold lysis buffer, and homogenized with a DUALL Kontes glass homogenizer (Kimble Chase, Vineland, NJ). For immunoblot analysis, equal amounts of liver (20 μg) or muscle (30 μg) homogenate from wild-type or rAAV-treated *Pah*^{enu2} mice were separated by 4–12% polyacrylamide gel electrophoresis (PAGE), blotted onto nitrocellulose membrane, and probed with rabbit anti-PAH antibody (diluted 1:10,000) or mouse anti-β-actin antibody (1:1000 or 1:10,000; Sigma-Aldrich, St. Louis, MO). The blots were incubated with a secondary antibody conjugated with peroxidase (1:10,000; GE Healthcare Biosciences, Piscataway, NJ) and the bands were visualized with enhanced chemiluminescence (ECL) solution (GE Healthcare Biosciences). Quantification of Western blots was done with NIH Image software, with the amounts of PAH protein normalized to the amount of β-actin protein detected.

AAV vector biodistribution

Real-time PCR analysis of genomic DNA extracted from snap-frozen tissues was performed with the same set of primers and probe indicated in the preceding section, Plasmid Vectors and Recombinant AAV Production. Details of the procedure have been described previously (Ding *et al.*, 2006a).

Statistical analysis

Statistical analyses were performed with GraphPad Prism software (GraphPad Software, San Diego, CA). Statistical differences between the various experimental groups were evaluated by *t* test. $p < 0.05$ was considered statistically significant.

Results

Dose response and kinetics of AAV serotype 1, 2, and 8 vector-mediated correction of murine PKU

To compare the efficacy of various AAV serotypes for mediating long-term therapeutic correction of PKU in *Pah*^{enu2} mice, we generated three different recombinant AAV2 genomes packaged with either serotype 1, 2, or 8 capsid. Each of these vectors contained an identical expression cassette consisting of the murine *Pah* cDNA under the control of the CMV enhancer–chicken β-actin (CBA) promoter with a woodchuck posttranscriptional regulatory element (WPRE) sequence. This expression cassette, named AAV2-PKU5, was previously described in our study of liver-directed gene transfer via intraportal or tail vein injection of recombinant AAV vectors (Ding *et al.*, 2006a). The AAV particles pseudotyped with serotype 1, 2, or 8 capsid (the vectors are named AAV2/1-PKU5, AAV2/2-PKU5, and AAV2/8-PKU5, respectively) were produced in the same manner to avoid inconsistencies due to differences in purification methodologies (Klein *et al.*, 2008). To evaluate the influence of the various AAV capsid proteins on long-term therapeutic correction, hyperphenylalaninemic male and female *Pah*^{enu2} mice were first injected with an equivalent, high dose of viral genomes (VG) of AAV2/1-PKU5 or AAV2/8-PKU5 vector (1.2×10^{12} VG/mouse) or with a slightly higher dose of 2.4×10^{12} VG/mouse for the comparative study with AAV2/2-PKU5. For each mouse, half of the total vector dose was administered to each hindleg by direct percutaneous injection into the gastrocnemius muscles. Given the low invasiveness of intramuscular injections, this route would be more advantageous for secondary gene transfer than intraportal delivery, and thus was selected for this study. Blood Phe levels were monitored periodically for several

weeks and used as a surrogate marker of transgene expression. In all treatment groups, blood Phe levels decreased significantly from levels greater than 1800 μM to less than 360 μM, the defined therapeutic value for blood Phe, by 2 weeks after vector injection, and this effect persisted in all injected mice, both males and females, for at least 10 weeks (Fig. 1). Normalization of Phe levels was also accompanied by a complete phenotypic change from brown hair to black at about 8 weeks, similar to results we (and others) have observed in previous studies (Ding *et al.*, 2006a, 2008). The duration of correction in males was monitored for more than 1 year and the results are summarized as follows: In males injected with the highest dose of vector AAV2/1-PKU5, AAV2/2-PKU5, or AAV2/8-PKU5, blood Phe levels were stably maintained at low levels for 54, 40, and 55 weeks postinjection, respectively. Blood Phe values rose slightly above 360 μmol/liter in some mice injected with AAV2/8-PKU5 at week 55, the last time point analyzed (Fig. 1c). Similarly, side-by-side comparative analyses were performed in females and the results showed striking differences in treatment effect between males and females, and among the three vectors tested. Although the normalization of blood Phe levels in females treated with the highest doses of AAV2/1-PKU5 and AAV2/8-PKU5 were temporary, they lasted out to 30 and 35 weeks postinjection, respectively (see Fig. 1a and c). Interestingly, blood Phe levels did not return to pretreatment values (:1800 μmol/liter), and all females remained only mildly hyperphenylalaninemic with Phe concentrations ranging from 700 to 900 μmol/liter up to 1 year. In female mice, AAV2/2-PKU5 treatment was least effective (Fig. 1b), as the Phe values in this cohort of mouse began to rise 10 weeks after gene transfer and returned to levels close to pretreatment values by 30 weeks.

Because these comparisons were made with relatively high vector doses, we wanted also to exclude the possibility that a potential vector hierarchy had been obscured because of saturating conditions. Therefore, we proceeded to test the long-term correction efficiency of the various AAV vectors in a dose range study. At a dose of 1.2×10^{11} VG/mouse, statistically significant correction in blood Phe was seen only in animals treated with AAV2/8-PKU5 vector (Fig. 1c) and not in mice receiving either AAV2/2-PKU5 or AAV2/1-PKU5. The therapeutic effect with AAV2/8-PKU5 vector, however, was only transient; blood Phe rose again to preinjection levels by 10 weeks postinjection. Only modest decreases in blood Phe were observed after treatment with 1.2×10^{11} VG of AAV2/1-PKU5 (Fig. 1a), 2.4×10^{11} VG of AAV2/2-PKU5 (Fig. 1b), or with a lower dose (1.2×10^{10} VG/mouse) of AAV2/8-PKU5 (Fig. 1c). In all cases, this modest effect lasted only a few weeks. As was expected, the results from the dose–response study confirmed that rAAV vector pseudotyped with serotype 8 capsid was the best performing vector. Surprisingly, however, at the highest dose, AAV serotypes 1 and 8 were almost equally effective in yielding long-term correction of blood Phe levels in both male and female *Pah*^{enu2} mice.

Tissue distribution of rAAV vector DNA

We anticipated that murine liver would be the principal target for transduction and PAH expression in our experiments because of the established general hierarchy of tissue-

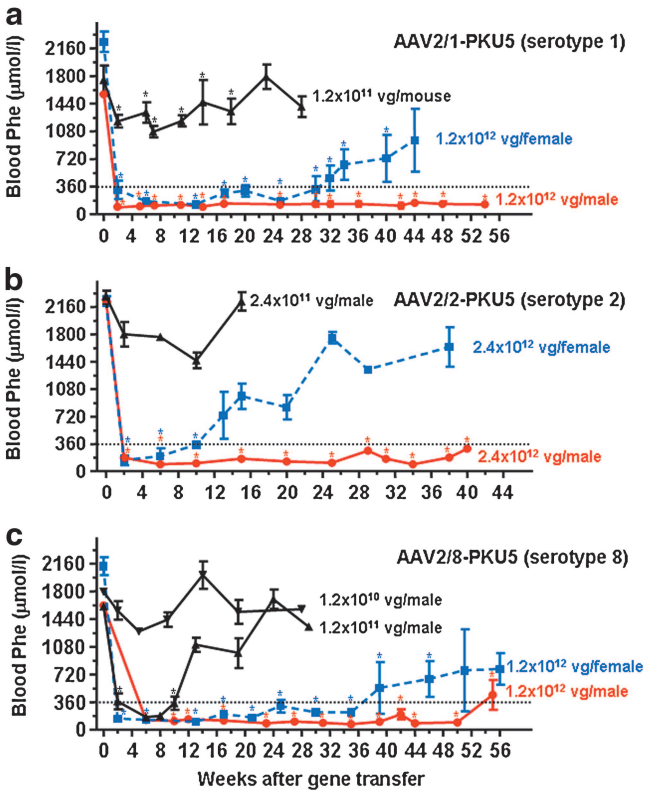

FIG. 1. Serotype-, dose-, and gender-dependent changes of blood phenylalanine (Phe) content in phenylketonuria (PKU) mice after intramuscular administration of vector AAV2-PKU5. Various doses of vector AAV2-PKU5 expressing phenylalanine hydroxylase (PAH) and pseudotyped with either capsid 1, 2, or 8 were injected into the gastrocnemius muscles of both hindlegs of male and female PKU mice. Approximately every second week blood was withdrawn from tail veins for Phe determination, depicted as a function of time after injection of AAV2-PKU5. Blood Phe concentrations are represented as means ± SD. The dotted lines in each graph indicate the therapeutic threshold of blood Phe concentration for treatment of patients with PKU (9360 μmol/liter). (**a**) Vector AAV2-PKU5 serotype 1 was injected at a dose of 1.2×10^{12} viral genome (VG) particles per mouse in males (red circles, $n=4$) and in females (blue squares, $n=3$), or at a dose of 1.2×10^{11} VG particles per mouse (black triangles, 3 males plus 3 females, $n=6$). (**b**) Vector AAV2-PKU5 serotype 2 was injected at a dose of 2.4×10^{12} VG particles per mouse in males (red circles, $n=2$), and in females (blue squares, $n=2$), or at a dose of 2.4×10^{11} VG particles for the lower dose–response study (black triangles, male, $n=3$). (**c**) Vector AAV2/8-PKU5 serotype 8 was injected at three different vector doses, in 10-fold increments, ranging from 1.2×10^{10} to 1.2×10^{12} VG particles per mouse. Data for males (red circles, $n=4$) and females (blue squares, $n=3$) are plotted separately for the 1.2×10^{12} VG particle dose–response study, whereas vector doses of 1.2×10^{10} VG particles (black inverted triangles, $n=3$) and 1.2×10^{11} VG particles (black triangles, $n=3$) were used for injection of two male groups. Blood Phe levels at various time points after AAV2-PKU5 injection were compared with preinjection blood levels by Student t test. Significant differences as compared with $t=0$ found for all AAV2-PKU5-treated animals are marked with asterisks, whereas at all unmarked points blood Phe was not significantly different from baseline levels ($p>0.05$).

Table 1. Vector Genome Copy Numbers per Cell in Selected Tissues

AAV vector (dose; time after injection; sex)	*Liver*	*Hindleg muscle*	*Foreleg muscle*	*Diaphragm*	*Heart*	*Kidney*	*Spleen*	*Brain*	*Lung*
AAV2/1-PKU5 (serotype 1)									
1.2×10^{12}; 54 weeks; M ($n = 4$)	279 ± 18	341 ± 18	0.4 ± 0.04	6 ± 0.2	20 ± 1	5 ± 0.3	3 ± 1	1 ± 0.2	3 ± 0.2
1.2×10^{12}; 56 weeks; F ($n = 1$)[a]	84 ± 6	232 ± 13	<0.1	1 ± 0.02	7 ± 0.1	0.3 ± 0.01	0.4 ± 0.03	2 ± 0.3	0.2 ± 0.01
1.2×10^{11}; 53 weeks; M ($n = 3$)	18 ± 0.1	75 ± 17	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
1.2×10^{11}; 53 weeks; F ($n = 2$)	10 ± 2	57 ± 6	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
AAV2/2-PKU5 (serotype 2)									
2.4×10^{12}; 55 weeks; M ($n = 2$)	96 ± 10	57 ± 9	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
2.4×10^{12}; 44 weeks; F ($n = 2$)	44 ± 10	49 ± 8	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
2.4×10^{11}; 38 weeks; M ($n = 3$)	2 ± 0.5	4 ± 1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
AAV2/8-PKU5 (serotype 8)									
1.2×10^{12}; 28 weeks; M ($n = 2$)	823 ± 80	184 ± 18	0.8 ± 0.06	1 ± 0.4	2 ± 0.05	0.2 ± 0.01	<0.1	<0.1	0.3 ± 0.10
1.2×10^{12}; 55 weeks; M ($n = 3$)	190 ± 16	109 ± 29	0.1 ± 0.5	0.4 ± 0.07	0.6 ± 0.07	<0.1	<0.1	0.6 ± 0.03	0.3 ± 0.14
1.2×10^{12}; 56 weeks; F ($n = 3$)	109 ± 6	79 ± 7	8 ± 0.3	1 ± 0.1	2 ± 0.1	1 ± 0.1	0.4 ± 0.08	0.2 ± 0.01	0.2 ± 0.01
1.2×10^{11}; 34 weeks; M ($n = 3$)	33 ± 2	9 ± 1	0.1 ± 0.01	<0.1	<0.1	<0.1	<0.1	<0.1	0.2 ± 0.004
1.2×10^{10}; 34 weeks; M ($n = 3$)	3 ± 1	2 ± 1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1

Abbreviations: AAV, adeno-associated virus; F, female; M, male; PCR, polymerase chain reaction; PKU, phenylketonuria; WPRE, woodchuck posttranscriptional regulatory element.

Persistence of viral genomes at various time points after intramuscular injection of AAV2-PKU5 serotypes 1, 2, and 8. Every tissue type was measured in triplicate. Genomic DNA was isolated from the indicated tissues and 100 ng of each was used as template for quantitative PCR analysis to determine vector copy numbers. Primers and probe for the WPRE sequence were designed with Primer Express (Applied Biosystems). Dilutions of the AAV2-PKU5 vector plasmids were used to generate standard curves. M, male; F, female; note that values less than 0.1 indicate numbers of 1 virally infected cell among 10 or more cells.

[a]A total of three mice was injected with vector AAV2/1-PKU5, and from these animals, one was kept for tissue distribution analysis while the other two were used for a second injection with vector AAV2/8-PKU5.

specific transduction efficiency of the various AAV serotypes (Wu *et al.*, 2006) and because the CBA promoter directs robust gene expression in the liver. Furthermore, our previous studies and those of others have demonstrated that tail vein administration of recombinant AAV vector particles leads to preferential transduction of the liver (Davidoff *et al.*, 2005; Ding *et al.*, 2006a). To confirm that hindlimb muscle injection of our vectors led to vector accumulation in the liver in addition to muscle deposition, and to further examine the influence of factors such as dose, gender, and serotype on vector biodistribution, AAV2-PKU5 vector genomes were quantified by qPCR analysis from identical amounts of genomic DNA extracted from liver, hindleg and foreleg muscles, diaphragm, heart, kidney, spleen, brain, and lung (see Table 1). In animals that had received the highest vector dose ($> 1.2\times10^{12}$ VG), AAV2-PKU5 vector DNA was detected in all tissues examined, but vector genome copies per cell (VG per cell) varied significantly between the samples. For all three AAV serotypes and in both male and female mice, most vector genomes were detected either in hindleg muscle tissue (the site of injection) or in liver. Muscle tissue from hindlegs of AAV2 serotype 1-injected mice contained the highest vector genome copy numbers per cell followed by the serotype 8- and 2-treated groups, as expected from several reports of preferential transduction of muscle tissue by rAAV2/1 vectors (Xiao *et al.*, 1999; Chao *et al.*, 2000; Arruda *et al.*, 2004; Louboutin *et al.*, 2005). Between weeks 28 and 56 postinjection of rAAV2/8 vector, the number of AAV2-PKU5 genomes measured in liver decreased roughly 8-fold whereas the number of genomes measured in the injected muscle decreased only by half. This difference may reflect the hepatocyte turnover rate and accelerated episomal vector clearance in liver in comparison with muscle. Administration of the highest dose ($> 1.2\times10^{12}$) of AAV vectors to female mice also led to efficient liver transduction, which was, however, 2- to 7-fold lower than the values found in the male cohort. The gender difference in vector genome copy numbers in liver was found to be highly significant for the AAV2 serotype 8-treated group ($p = 0.0013$ by Student t test). Such sex-dependent differences in transduction of murine liver by AAV has also been reported by other groups (Davidoff *et al.*, 2003) (see also Discussion). A gender difference for muscle (hindleg) transduction with vectors serotype 1 and 8 was also observed, but this difference was much less pronounced than for liver. The gender difference in vector genome copy numbers per cell for all three AAV serotypes is also reflected in the difference for long-term Phe clearance as shown in Fig. 1. In contrast to liver, the difference in hindleg muscle transduction for both AAV2 serotype 1 and 8 vectors seemed to be slightly less pronounced between females and males, with values in females decreasing by only approximately one-third to one-fourth compared with copy number in males. Similar gender-related differences were noted at lower vector doses (Table 1). The biodistribution of these vectors in all other tissues, that is, foreleg muscle, diaphragm, heart, kidney, spleen, brain, and lung, was also influenced by the AAV capsid proteins. The overall results suggested a gender-related difference in vector distribution especially observable for the serotype 8 vector.

Tissue PAH activities

In general, the transduction profile found in liver or muscle was also reflected in the tissue PAH activity (Table 2). Most notably, the results indicated that liver PAH activities ranging from 0.25 to 0.58 mU/mg, equivalent to 10–23% of wild-type liver PAH activity, were sufficient to maintain blood Phe levels well below the curative threshold. Furthermore, for some mice, values of 0.10 mU/mg, equivalent to 4% wild-type liver PAH activity, were associated with Phe concentrations between 742 and 839 μmol/liter, which are considered to be characteristic levels of mild human hyperphenylalaninemia. Unfortunately, the relationship between wild-type protein levels and liver PAH activity could not be assessed through immunoblotting because of indiscriminate detection of both wild-type and mutant PAH proteins in livers of *Pah*^{enu2} mice (Ding *et al.*, 2004) and insufficient amounts of vector-mediated PAH expression to enable relative quantitation of expression levels between treated and untreated *Pah*^{enu2} mice (data not shown). In contrast, the results from Western blot analysis of vector-injected muscles demonstrated a direct correlation between vector genome copy number and amount of detectable PAH protein (data not shown).

Administration of rAAV2/8 after prior injection with rAAV2/1 vector

Several studies have reported strategies to increase or sustain transgene expression levels through serial injection of rAAV vectors with alternative capsid serotypes to avoid immunity against previously administered viral vectors (Halbert *et al.*, 2000; Riviere *et al.*, 2006; Nathwani *et al.*, 2009). Because rAAV-treated *Pah*^{enu2} females were more reluctant to achieve long-term therapeutic correction than males, we sought to examine whether it might be possible to further extend the therapeutic duration by administering an alternative serotype vector. For this purpose, two *Pah*^{enu2} females, which had been previously exposed to 1.2×10^{12} AAV2/1-PKU5 vector genomes and with blood Phe values rising to pretreatment levels by week 44 (Fig. 1a), were reinjected intramuscularly with an equivalent dose of serotype 8 vector (1.2×10^{12} VG of AAV2/8-PKU5 per mouse; see Fig. 2). Blood Phe concentrations subsequently decreased from 1260 ± 631 μmol/liter to therapeutic levels (<360 μmol/liter) by 3 weeks after secondary injection. Blood Phe remained at therapeutic levels up to 13 weeks after the second injection but increased thereafter to reach values of mild hyperphenylalaninemia (713.2 ± 216 μmol/liter). It should be noted, however, that in comparison with females only injected once with vector AAV2/8-PKU5 and in which therapeutic Phe levels were maintained up to 35 weeks (Fig. 1c), the therapeutic effect of AAV2/8-PKU5 treatment was less persistent in animals receiving secondary injections. This observation suggests that the transduction efficiency of secondary gene transfer is likely to be influenced or affected by the delivery of the first vector or by the age at which the animals were treated the second time. Nonetheless, these results demonstrate a successful method to prolong therapeutic correction in female mice to a similar duration as was achieved in male mice.

Effect of BH_4 supplementation on blood phenylalanine in rAAV-treated female mice

We have previously reported that the BH_4 cofactor stimulates and protects the activity of normal murine PAH in

Table 2. Phenylalanine Hydroxylase Activities in Tissues of Liver and Muscle, and Liver Tetrahydrosiopterin Content of AAV2-PKU5-Injected Hindleg Muscle of PKU Mice in Comparison with Controls[a]

Sex of (PKU) mouse and AAV treatment	*Time after gene transfer (weeks)*	*Blood phenylalanine*[b] *(μmo/liter)*	*Liver PAH activity (mU/mg)*	*Liver BH_4 content (pmol/mg)*	*Muscle PAH activity*[c] *(mU/mg)*
Controls					
Male BL/6 wild-type untreated ($n = 4$)	>12 (adult)	<120	2.51 ± 0.32	26.64 ± 2.63[d]	<0.02
Male or female PKU untreated[e] (BL/6-*Pah*^{enu2})	>12 (adult)	1695 ± 385 (males)[d] 2175 ± 194 (females)	<0.02[d]	43.77 ± 3.38[d]	<0.02[d]
AAV2/1-PKU5 (serotype 1)					
Male PKU ($n = 4$) (untreated foreleg)	54	129 ± 49	0.35 ± 0.09	23.89 ± 0.97	0.58 ± 0.15 (0.03 ± 0.01)
Female PKU[e] ($n = 1$) (untreated foreleg)	56	752 ± 10	0.11 ± 0.01	18.42 ± 3.93	0.29 ± 0.04 (0.05 ± 0.01)
AAV2/2-PKU5 (serotype 2)					
Male PKU[e] ($n = 2$) (untreated foreleg)	55	769 ± 56	0.10 ± 0.09	21.56 ± 1.87	0.06 ± 0.01 (ND)
Female PKU[e] ($n = 2$) (untreated foreleg)	44	1350 ± 120	0.09 ± 0.03	ND	0.05 ± 0.01 (ND)
AAV2/8-PKU5 (serotype 8)					
Male PKU ($n = 2$) (untreated foreleg)	28	103 ± 12	0.58 ± 0.22	17.77 ± 1.21	0.19 ± 0.04 (0.03 ± 0.01)
Male PKU ($n = 2$) (untreated foreleg)	55	210 ± 30	0.25 ± 0.09	16.48 ± 0.32	0.17 ± 0.04 (0.08 ± 0.01)
Male PKU[e] ($n = 1$) (untreated foreleg)	55	839 ± 56	0.10 ± 0.01	25.36 ± 1.63	0.11 ± 0.03 (0.03 ± 0.01)
Female PKU[e] ($n = 1$) (untreated foreleg)	56	747 ± 87	0.11 ± 0.02	24.69 ± 1.50	0.36 ± 0.06 (0.05 ± 0.02)

Abbreviations: BH_4, tetrahydrobiopterin; ND, not determined; PAH, pherylalanine hydroxylase.
[a]All measurements done in triplicate.
[b]Last blood phenylalanine values shortly before euthanasia of treated PKU mice.
[c]AAV2-PKU5-injected hindleg muscle; values in the untreated foreleg muscle are given in parentheses.
[d]Numbers were determined in a previous report (see Table 1 in Ding *et al.*, Mol. Ther. 16:673–681, 2008).
[e]Mice with hyperphenylalaninemia.

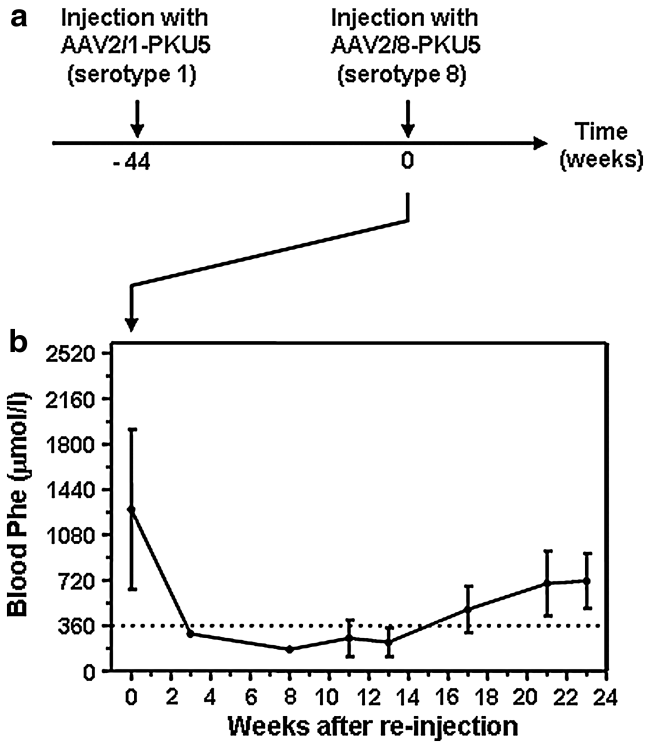

FIG. 2. Time course of blood Phe levels in PKU female mice after readministrations of AAV2-PKU5 vectors pseudotyped with alternative serotypes 1 and 8. (**a**) Time diagram for the intramuscular readministration experiment using alternative serotypes. At time point −44 weeks, 1.2×10^{12} particles of vector AAV2/1-PKU5 (serotype 1) was injected into both hindleg muscles of PKU females ($n=3$). Forty-four weeks later, indicated as time 0 in the graph, two females were subjected to a second injection with 1.2×10^{12} viral particles of vector AAV2/8-PKU5 (serotype 8). (**b**) Blood Phe values of PKU female mice after reinjection. At each time point the result is represented as the mean ± SD. The dotted line indicates the threshold for Phe concentration at the therapeutic level (9360 µmol/liter).

liver (Thöny *et al.*, 2004; Scavelli, 2006; Pey *et al.*, 2008). Moreover, expression of PAH in skeletal muscle in the presence of adequate BH_4, or coexpression of PAH along with the essential BH_4 biosynthetic genes, can effectively clear Phe from the circulation of PKU mice (Ding *et al.*, 2008). We reasoned that as liver PAH activity was lost in rAAV-treated female mice, Phe clearance could be additionally limited by relative BH_4 cofactor deficiency. To test this hypothesis, we supplemented BH_4, using a single dose of synthetic cofactor, by intraperitoneal injection of 16 mg of BH_4 per kilogram body weight, an amount that is comparable to the recommended oral loading tests in human patients, that is, 20 mg of BH_4 per kilogram of body weight. Supplementation of BH_4 was done for both male and female mice 38 weeks after administration of rAAV2/2-PKU5 vector (2.4×10^{11} VG/male mouse and 2.4×10^{12} VG/female mouse). Blood Phe was measured before and 6, 12, and 24 hr after BH_4 injection. As shown in Fig. 3a, blood Phe in rAAV-treated males was not affected by BH_4 supplementation. However, in female rAAV2/2-PKU5-treated mice, blood Phe concentrations decreased transiently in response to BH_4 injection. Successful Phe clearance after BH_4 treatment was also observed for mice previously injected with the highest rAAV2/8-PKU5 dose (Fig. 3b). To further assess whether skeletal muscle tissue, which lacks endogenous cofactor production, could participate in Phe clearance, BH_4 responsiveness was compared between male mice injected with a low dose of rAAV2/1-PKU5 (1.2×10^{11} VG/mouse; at week 53) and rAAV2/8-PKU5-treated females from the high-dose cohort group (1.2×10^{12} VG/mouse; at week 56; see Table 1). Retrospectively, the number of vector genomes measured in DNA recovered from hindleg muscle was similar in these two groups (75 ± 17 VG/cell in males compared with 79 ± 7 VG/cell in females), but the number of vector genomes in liver was at least 6-fold lower in the rAAV2/1-PKU5-treated group than in the rAAV2/8-PKU5-treated mice (18 ± 0.1 VG/cell for rAAV2/1-PKU5 vs. 109 ± 6 VG/cell for rAAV2/8-PKU5). In contrast to the rAAV2/8-PKU5-treated mice, loading with BH_4 either by a single intraperitoneal injection or by repeated oral administration for 5 days (32 mg of BH_4 per kilogram body weight per day) did not result in a decrease in blood Phe levels in rAAV2/1-PKU5 mice (data not shown). These results indirectly show that in our setting only liver, not muscle, PAH activity profits from BH_4 cofactor treatment.

We subsequently addressed further the possibility that BH_4 content in the livers of treated animals could be a limiting factor for Phe clearance; we therefore investigated directly the effect of exogenously administered cofactor on residual PAH activity. As shown in Fig. 4a, no significant difference in liver BH_4 levels before BH_4 supplementation was found between successfully corrected rAAV-injected males, partially corrected rAAV-injected females, or wild-type females ($p>0.05$ by one-way analysis of variance [ANOVA] followed by Bonferroni's post hoc test). To confirm that BH_4 was effectively taken up into liver and muscle after intraperitoneal injection, we measured BH_4 content in tissues of rAAV-treated animals (Fig. 4b). Twenty-four hours after BH_4 administration by intraperitoneal injection, BH_4 content had increased more than 5-fold in liver over pretreatment levels in females injected with rAAV2/8-PKU5 vectors (Fig. 4a and b) and was almost 4-fold higher in muscle of BH_4-treated mice in comparison with wild-type mice that had not received BH_4 (Fig. 4b). Similar results were seen in male mice that had been treated with rAAV2/8-PKU5 (Fig. 4b). BH_4 supplementation in both female and male rAAV-treated mice was associated with a 3.3- and 2.3-fold increase, respectively, in measured liver PAH activity compared with untreated *Pah*^{enu2} animals. The increased PAH activity was apparently sufficient to allow therapeutic Phe clearance in these mice (see Figs. 3b and 4c). A 1.7-fold increase in PAH activity on BH_4 treatment was also found in hindleg muscle from both genders (Fig. 4d). To further assess the direct effect of BH_4 supplementation on PAH, immunoblotting and densitometric quantification of PAH protein were performed. In liver of rAAV2/8-PKU5 treated mice, we observed an ~2-fold increase in PAH protein levels after BH_4 loading (Fig. 4e). In contrast to rAAV-transduced liver, where BH_4 might have stabilized both endogenous mutant PAH monomers and wild-type PAH protein expressed from the vector transgene, muscle homogenate containing only transgenic wild-type PAH protein was used to assess the direct relationship between PAH protein levels with and

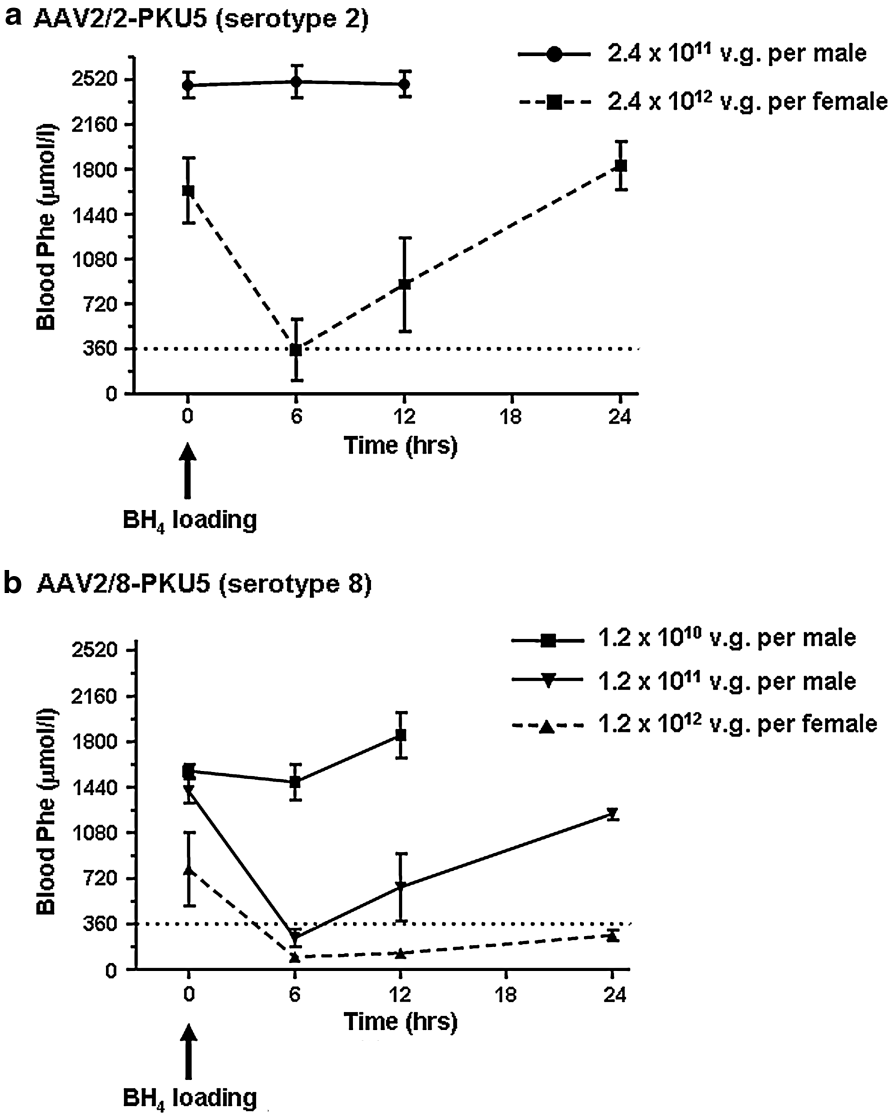

FIG. 3. Effect of BH_4 supplementation in AAV2/2-PKU5- and AAV2/8-PKU5-treated PKU mice. PKU mice that underwent AAV vector administration were given a single intraperitoneal injection of BH_4 either when their blood Phe levels started to rise again and nearly reached pretreatment levels, or when they had only a marginal decrease in Phe levels but no therapeutic correction. Mean Phe levels ± SD before BH_4 administration versus 6, 12, and 24 hr posttreatment are plotted (dotted line represents the Phe level of 9360 μmol/liter). (**a**) Mean blood values for Phe values versus time in AAV2/2-PKU5 (serotype 2)-treated PKU males (circles, $n = 3$) and female mice (squares, $n = 2$) after a single intraperitoneal injection of BH_4. BH_4 injections were performed 38 weeks after gene transfer in males and females previously injected with 2.4×10^{11} and 2.4×10^{12} particles of vector AAV2/2-PKU5 (serotype 2), respectively. (**b**) Same as in (**a**), but mice were pretreated with vector AAV2/8-PKU5 (serotype 8): squares, males ($n = 3$) 28 weeks after gene transfer of 1.2×10^{10} VG; inverted triangles, males ($n = 3$) 34 weeks after gene transfer of 1.2×10^{11} VG; triangles, females ($n = 2$) 56 weeks after gene transfer of 1.2×10^{12} VG.

without BH_4 supplementation. As shown in Fig. 4f, slightly higher PAH protein levels were detected in both hindleg and foreleg muscle homogenates on BH_4 treatment compared with untreated females, that is, a 1.3-fold increase by densitometric analysis for the hindleg and a 20-fold increase for foreleg muscles. In rAAV2/8-PKU5-treated males, which received a 10-fold lower virus dose compared with the female group, PAH protein levels were at or slightly below the detection limit of our antibody, as we detected PAH protein only in hindleg muscle of animals supplemented with BH_4.

Taken together, these results suggest that the liver PAH activity in treated *Pah*^{enu2} animals, which had not led to efficient removal of circulating Phe, could be increased by exogenous BH_4 supplementation. In contrast, heterologous expression of PAH in muscle tissues with or without BH_4 administration did not result in blood Phe clearance. Because in the BH_4-treated mice a 10- to 15-fold lower amount of BH_4 was found in muscle tissues compared with liver, the rate of BH_4 uptake in muscle tissues could have been the limiting factor in the clearance of circulating Phe. The slow rate of BH_4 uptake in muscle in comparison with liver has been reported previously (Harding *et al.*, 1998, 2004). Alternatively, the level of PAH protein expression in muscle of rAAV-treated mice could have been insufficient for effective blood Phe clearance even after supplementation with BH_4.

Discussion

Recombinant AAV gene delivery vectors, owing to the variety of applications and tissue types toward which these vectors may be targeted, hold great promise for the treatment of a multitude of genetic disorders and acquired human disease (Alexander *et al.*, 2008). Several studies have shown that administration of rAAV vectors to a variety of animal models including mice, dogs, and nonhuman primates can result in long-term gene expression and disease correction with minimal toxicity (Kootstra and Verma, 2003). These efforts have culminated in various clinical phase I–II studies with AAV2 serotype 2 (Edelstein *et al.*, 2007). Regardless of the success with these approaches, gene therapy correction in those models is currently limited to a period of 1 year or longer because of progressive decline in transgene expression over time, indicating that vector readministration may be necessary at intervals of several years. Furthermore,

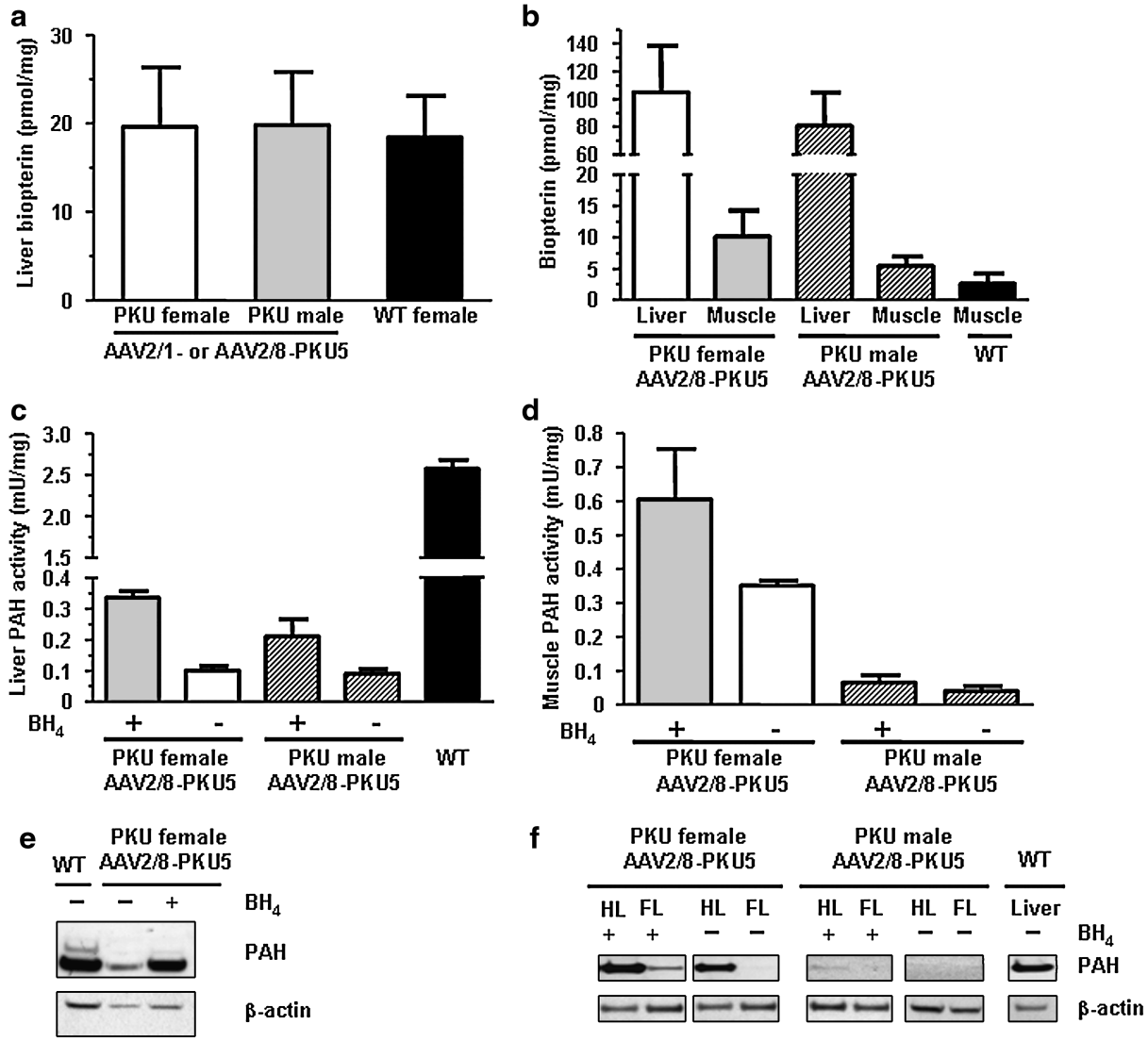

FIG. 4. Effect of BH_4 supplementation on PAH in AAV2-PKU5-treated mice. BH_4 content and PAH activity are indicated as means ± SD. Note that all AAV2/8-PKU5-treated female PKU mice were at week 56 after gene transfer of 1.2×10^{12} VG injected, and all AAV2/8-PKU5-treated male PKU mice were at week 34 after gene transfer of 1.2×10^{11} VG injected. (**a**) Total hepatic BH_4 content was analyzed in two groups of AAV2-PKU5-treated PKU male ($n = 7$) and female ($n = 7$) mice, and compared with levels of normal wild-type females (WT; $n = 5$). For both groups of virus-treated PKU mice, the liver BH_4 content of four AAV2/1-PKU5-treated animals and three AAV2/8-PKU5-treated animals was analyzed. (**b**) Total BH_4 content in liver and skeletal muscle of females ($n = 2$) and males ($n = 2$), both treated with rAAV2/8-PKU5 vectors, 24 hr after intraperitoneal injection of synthetic cofactor (1 μmol/g body weight in 1% ascorbic acid). The black column indicates the BH_4 content in muscle extracts of normal mice as control (not injected with synthetic BH_4; $n = 3$). (**c** and **d**) PAH enzyme activities in liver and muscle extracts, respectively, in the same animals as shown in (**b**), that is, 24 hr after BH_4 treated ($n = 2$) compared with non-BH_4-treated ($n = 1$) AAV2/8-PKU5-injected mice. The black column in (**c**) indicates PAH activity of normal mouse liver extracts (WT; $n = 3$). Note that muscle PAH activity between the sexes is different in (**d**), as males received a 10-fold lower dose of AAV vectors than females. (**e** and **f**) Western blot analyses of liver and muscle extracts, respectively, from the same BH_4-treated and untreated mice. In (**e**) equal amounts of liver homogenate (20 μg) from a wild-type mouse (WT) compared with AAV2/8-PKU5-treated PKU female mice with (+) or without BH_4 treatment (−) were probed with rabbit anti-mPAH antibody (diluted 1:10,000). In (**f**) each lane contains 30 μg of homogenized hindleg (HL) or foreleg (FL) muscle homogenate from animals treated with AAV2/8-PKU5 vectors after treatment with BH_4 (+) or no treatment (−). Thirty micrograms of liver homogenate of a wild-type mouse is shown as a control (WT). *Bottom:* Representative loading control using an anti-β-actin antibody, diluted 1:10,000 in (**e**) and 1:1000 in (**f**).

sex-related differences in biodistribution, differences in transduction efficiency, the lack of identified cell receptors for many rAAV serotypes, timing of expression, and levels of transgene expression observed in many preclinical experiments with AAV vectors are other unsolved issues that may critically impact the outcome of a gene transfer approach (Davidoff *et al.*, 2003; Berraondo *et al.*, 2006; Ogura *et al.*, 2006; Wu *et al.*, 2006; Buning *et al.*, 2008; Ho *et al.*, 2008). Thus, the development and investigation of multiple or combined treatment strategies may offer alternative options

to further extend the therapeutic effect required and address the issue of gender-dependent difference in transgene expression.

We have reported two different approaches to achieve long-term correction of hyperphenylalaninemia in a PKU mouse model. In the first approach, liver-directed gene transfer with the recombinant AAV2-PKU5 pseudotype 8 vector was explored, expressing the PAH transgene from the CBA promoter (Ding *et al.*, 2006a). Single doses of up to 10^{13} viral particle vectors administered by intraportal or tail vein injection result in therapeutic correction lasting for up to 1 year for males and females. In a second approach, single doses of approximately 10^{12} viral particles of a triple-cistronic AAV pseudotype 1 vector encoding murine PAH along with two BH_4-biosynthetic enzymes expressed from a CMV promoter were employed for muscle-directed gene transfer; this approach was associated with therapeutic blood Phe clearance for up to 70 weeks in our mouse model (Ding *et al.*, 2008). In both approaches, the therapeutic effect in some mice persisted for 1 year or longer, whereas other animals, in particular females, exhibited rising blood Phe levels at earlier time points. Results from the biodistribution analysis in the muscular approach study also indicated that some dissemination of vectors occurred to other tissues with preferential accumulation in the liver, an observation that has been reported by others (Manno *et al.*, 2003; Asokan *et al.*, 2008). On the basis of this last observation and our previous studies, we believe that long-term correction of hyperphenylalaninemia may further be strengthened by a concomitant muscle- and liver-targeted gene transfer approach via intramuscular injection. In the present study, we evaluated the feasibility to effect long-term therapeutic correction of PKU by intramuscular injection-mediated liver transduction with various alternative rAAV serotypes as a preliminary assessment toward multiple combined approaches. The choice of AAV serotypes and the intramuscular administration route were selected for these experiments because (1) liver as well as skeletal muscle have been successfully transduced with rAAV pseudotype 8 in various animal models (Sarkar *et al.*, 2004; Wang *et al.*, 2005; Inagaki *et al.*, 2006), and (2) rAAV2/8-mediated liver transduction is now being considered for treatment of patients with hemophilia B (Arruda *et al.*, 2004). Similarly, rAAV pseudotype 1 is reportedly the most efficient vector for muscle transduction in mice (Hauck and Xiao, 2003; Louboutin *et al.*, 2005). rAAV serotype 2 was also included in this study, being not only the most well-characterized AAV serotype with modest tropism for liver and muscle, but also having been used in clinical phase I–II trials (Rabinowitz *et al.*, 2002). To ensure that differences between the vectors used here were limited to the three different capsids, the rAAV2-PKU5 pseudotype 1, 2, and 8 vectors were purified and concentrated in parallel by an identical CsCl-density ultracentrifugation-based procedure. We found that all three vector serotypes were able to direct efficient clearance of serum Phe for at least 10 weeks for males and females after intramuscular injection of doses of 1.2×10^{12} for pseudotypes 1 and 8, or a dose of 2.4×10^{12} for serotype 2. On the basis of vector biodistribution and PAH activities determined in diverse tissues (see Tables 1 and 2), significant amounts of viral vector genome were found only in liver and hindleg muscles. The pseudotype 8 vector was detected primarily in liver and pseudotype 1 in hindleg muscle, and the relatively larger drop over time of vector copy number in liver compared with hindleg muscle may indicate a higher turnover rate in liver and more stable maintenance of vector in muscle tissue (Table 1). Furthermore, loss of episomal viral genomes is thus a highly probable explanation for the decrease in Phe clearance observed over time in our experiments. Immunologic rejection of transduced hepatocytes or myocytes has not been formally ruled out, but we have never detected anti-PAH antibodies in the blood of rAAV-treated mice and the histologic examination of transduced tissues was normal without any evidence of lymphocyte infiltration (Ding *et al.*, 2006a, 2008). Interestingly, the results from the high dose–response experiments showed that rAAV2-PKU5 with pseudotypes 1 and 8 were almost equivalent in mediating long-term Phe clearance with persistently low blood Phe levels for up to 1 year in males, whereas females were corrected for at least 30 to 35 weeks after gene transfer.

The interaction between host and vector in the context of gene therapy is complex and seems to vary considerably according to vector type, serotype, dose, volume of injection, and route of administration, as well as the species being studied. This complexity also comes into play when trying to interpret our finding. Evaluation of vector biodistribution in this study did not provide direct evidence of rAAV vectors in the blood, but results from preclinical and clinical reports evaluating rAAV2/1 and rAAV2/8, as well as intramuscular delivery protocols, suggest that leakage of vector from the injection sites is likely to occur via the circulation. Kinetic studies, for instance, have shown that the number of infectious particles in the blood of mice or larger animals injected with rAAV was maximal at 6 hr and accounted for 5% of the total dose in one study and a 7-log less concentration of the total injected dose in another study (Flotte *et al.*, 2007; Toromanoff *et al.*, 2008). The appearance of vector DNA in blood was followed by rapid clearance to low or undetectable levels within 7 to 14 days after the injection and similar outcome has also been observed in two clinical trials (Brantly *et al.*, 2009; Mingozzi *et al.*, 2009). These observations, along with the extensive vascularization of skeletal muscle, would favor AAV dissemination via the systemic circulation to other organs such as the liver. However, the presence of viral vector in draining and distant lymph nodes of animals injected intramuscularly suggests that the lymphatic system may participate in the dissemination of viral vector as well (Rip *et al.*, 2005).

Notwithstanding that the selection of an appropriate capsid serotype is of pertinent importance to deliver AAV vectors to specific target tissues, the intrinsic interactions between various serotypes and muscle tissues could also have affected the amount of vector that went into the blood and then transduced the liver. It is likely that any serotypes that would be efficiently internalized by muscle myofibers could lead to less transduction of remote sites owing to the low number of infectious particles gaining access to the blood. This might explain why the injection of a low dose of serotype 1, which is known to transduce muscle well, was not as efficient as serotype 8 in transducing hepatocytes. Data also suggest that rAAV2/1 can be viewed as a serotype with low intramuscular diffusion abilities, leading to high transgene expression at the intramuscular injection site versus low expression in the surrounding area (Toromanoff *et al.*,

2008). Thus, with high-dose vector injection it might be possible that local saturation of serotype 1 vector internalization may have resulted in a higher concentration of viral particles circulating in the blood and thus better liver transduction. On the other hand, a differential rate of dissemination might also be explained by the relative amount of cellular receptor and its affinity for a particular serotype. Strong binding to heparan sulfate proteoglycans, which are abundantly expressed on the surface of muscle cells, might decrease the dissemination of AAV2/2 vector from the site of administration (Asokan *et al.*, 2008). In addition, it has also been hypothesized that some specific serotypes, such as AAV1, in certain tissues such as skeletal muscle may be trapped as intact infectious particle in "protective" locations such as the transverse tubules and could then be slowly released over time into the systemic compartment (Toromanoff *et al.*, 2008).

AAV vectors were also delivered intramuscularly with a total injection volume of 50 μl administered to each hindlimb. This injection volume in a 30-g mouse would be equivalent to injecting 130 ml into the muscle of an 80-kg human. Certainly, the injection volume and the resulting hydrostatic pressure in the injected muscle body could influence the amount of vector that gains access to the vasculature and is transported to the liver. We have not investigated this variable in detail. Reportedly, intramuscular injection of human α_1-antitrypsin (AAT)-expressing rAAV2/8 vector in a 0.4-ml volume into mice yielded 9-fold higher serum hAAT levels than in mice receiving the same vector dose but concentrated in 0.01 ml (Ye *et al.*, 2009). In human clinical trials with intramuscular administration of AAV vectors, total doses of 10–20 ml divided into 0.3- to 0.5-ml injections were delivered to multiple injection sites. Administration of 20 ml to an 80-kg person would be equivalent to injecting only 8 μl into a 30-g mouse. Despite the relatively low injection volume used, AAV vector administered to muscle has been detected in the blood of human subjects treated in clinical trials (Manno *et al.*, 2003).

We witnessed a time-dependent loss of therapeutic effect after rAAV injection in some animals, particularly in female mice. BH_4 supplementation by intraperitoneal injection transiently improved Phe clearance but only in those mice that had retained some minimal amount of liver PAH activity. The molecular mechanisms that mediate BH_4 responsiveness are not yet completely elucidated, but several *in vitro* and *in vivo* studies support the hypothesis that BH_4 plays a role in the protection of PAH protein against degradation (Thöny *et al.*, 2004; Scavelli *et al.*, 2005). The therapeutic effect of rAAV treatment was much longer lasting in male mice than in female mice with all vector pseudotypes and doses. The reasons for this difference have not been completely elucidated. Second injection of rAAV vector with alternative capsid pseudotypes into previously treated female mice did lead to transient restoration of blood Phe to therapeutic levels. However, the duration of this effect was shorter in animals receiving secondary injections than in naive mice injected for the first time. The cause for this discrepancy is yet unknown. Possible explanations include an immunologic response arising after the first injection that then cross-reacts with the second vector injected. Other investigators have also shown that initial treatment with AAV serotype 2 vectors led to a 20-fold impairment of subsequent rAAV pseudotype 1-mediated transduction (Xiao *et al.*, 1999). The extent of this impairment seems dependent on the species treated, the route of vector administration, and the specific vector pseudotype. We cannot exclude the possibility that the age of the mice at the time of the secondary injections may have influenced the effectiveness of the treatment. Our experiment confirmed the previously reported observation that transduction frequency after rAAV administration is gender dependent, especially for rAAV2/8-mediated liver transduction, but we did not detect a gender difference in hepatic BH_4 cofactor content, a subject of previous conjecture (Chen *et al.*, 2007).

The animal model used here for PKU obviously has species limitations related to assessing function, immune response, and distribution of PAH. Because a primate model for the disease is not available, at this point it is difficult to design preclinical studies that accurately predict clinical outcomes. Moreover, animals such as mice cannot provide data directly applicable to human subjects, especially on safety and immunologic profiles. Another critical issue in translation to human patients involves vector delivery across the vascular barrier. Although this has been achieved in the PKU mouse by using AAV in this and our previous works (Ding *et al.*, 2006a, 2008), successfully translating rodent studies to patients remains a challenge. Furthermore, dosing issues are also paramount in scaling gene delivery from small animal to human patients. In the absence of a large animal model for PKU, preclinical studies will surely necessitate the use of therapeutic transgenic surrogate genes such as luciferase, green fluorescent protein (GFP), or a more relevant marker such as, for example, a PAH-FLAG-tagged protein.

In summary, intramuscular injection of rAAV vectors, the relatively noninvasive gene transfer strategy used in this study, is as effective as our previous intravenous vector administration (Ding *et al.*, 2006) in producing therapeutically relevant levels of liver PAH expression and successful correction of Phe clearance in an animal model of human PKU. However, and in the light of the dose-dependent immune toxicity and rAAV-related liver genotoxicity (Manno *et al.*, 2006; Zaiss and Muruve, 2008), it is perhaps critical to emphasize that in this study a 17-fold lower dose of rAAV2/8-PKU5 vector was used than in our previous report on liver-directed gene transfer via the portal vein and tail vein. BH_4 supplementation or secondary injection of an rAAV vector with an alternative capsid pseudotype can help sustain liver PAH activity as PAH expression wanes over time. Perhaps the future development of rAAV vectors that mediate site-specific integration of the therapeutic transgene into the host genome will lead to sustained liver PAH expression. Gene therapy using the phenylalanine ammonia lyase (PAL) enzyme expressed from a viral or nonviral system might be an alternative treatment approach. Results from preclinical and clinical phase I studies using PAL as an enzyme substitution therapy are encouraging and, further, PAL does not require any exogenous cofactors (Sarkissian and Gamez, 2005; Sarkissian *et al.*, 2008, 2009; Kang *et al.*, 2010). All these studies have the goal to develop a safe and efficient gene transfer method for the long-term treatment of human PKU.

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Author Disclosure Statement

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